# MULTIPLE DEOXYRIBONUCLEASE ACTIVITIES IN NUCLEI OF HeLa CELLS[1]

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SUMMARY: Extracts of purified HeLa nuclei contain DNase activities which can be separated by CM-Sephadex column chromatography into four distinct enzymes. These DNases differ in their resistance to thermal inactivation and relative activity on different substrates. Each of these DNases can increase the template-primer activity of native DNA for DNA polymerase action.

## INTRODUCTION

The enzymes involved in mammalian excision repair, or the putative "nickases" which are thought to initiate DNA replication, have not been positively identified. Inasmuch as these enzymes act in the cell nucleus, it might be expected that they should be associated with the nuclei, if only at some stage of the cell cycle. Indeed, various DNases have been found in the nuclei of eukaryotic cells. Several laboratories reported that a DNA endonuclease is associated with nuclei from rat liver (1, 2, 3), mouse liver (4) and sea urchin embryo (5), while in rabbit bone marrow two DNA-specific exonucleases were found to be localized predominantly in the nuclei (6, 7). Calf thymus nuclei contain a nuclease with properties of DNase II (8).

We wish to expand on our earlier report that nuclei of HeLa cells exhibit DNase activity (9), by reporting the extraction and partial purification of four distinct DNases.

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## MATERIALS AND METHODS

Tissue Culture: HeLa S3 cells were propagated as described previously (10). Mycoplasma contamination was excluded by tests performed every 7-14 days (11).

Nuclear Isolation and Extraction: Nuclei were isolated from 6 x $10^9$ exponentially growing cells by a method shown by electron microscopy to produce HeLa nuclei free from cytoplasmic contamination (12). The nuclei were extracted and the extract fractionated as summarized in Fig 1. All procedures were performed at 4°C. The three fractions obtained were applied separately to columns (0.9 cm x 20 cm) of CM-Sephadex (Pharmacia, Piscataway, N.J.). The columns were washed with equilibration buffer (50 mM potassium phosphate pH 7.0, 1 mM $Na_2$ EDTA, 1 mM DTT, 40% ethylene glycol) and developed with a linear 0-0.5 M NaCl gradient. The chloride content was measured by a Buchler-Cotlove chloridometer.

Enzyme Assays: DNase activity was measured in three ways: a) by optical density readings of the acid soluble hydrolysis products from heat denatured (100°C, 20 min) calf thymus DNA (Worthington); b) by the extent of hydrolysis of DNA-$^3$H isolated from E. coli by the method of Marmur (13) (SA 5.2 x $10^6$ dpm per nucleotide equivalent); c) by the extent of hydrolysis of poly d(A-T)-$^3$H (Miles, SA 25 $\mu$Ci per $\mu$mole P); and d) by the ability of DNase to increase the priming efficiency of calf thymus DNA in a subsequent DNA polymerase (M. lysodeikticus, Sigma) reaction (14).

RNase activity was estimated using poly rC-$^3$H (Miles, SA 51.5 $\mu$Ci per $\mu$mole P) in the same manner as poly d(A-T)-$^3$H with the use of 6 mM uranyl nitrate - 0.4 N $HClO_4$ as precipitant.

DNA polymerase was assayed by the method of Loeb (15).

Native calf thymus DNA (2 mg/ml 0.02 M KCl) was irradiated for 30 min at a distance of 38 cm with a General Electric germicidal lamp to obtain UV-irradiated DNA.

## RESULTS

DNases extracted from highly purified HeLa nuclei eluted from CM-Sephadex[4] in four distinct regions (Figs 2-4). The enzyme, which was extracted from the nuclei at high ionic strength, and therefore, probably a component of chromatin, did not bind to CM-Sephadex and was found in the buffer wash (Fig 2). We shall refer to this activity as DNase A.

The easily extractable, probably nucleoplasmic, enzymes were

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[4]Abbreviations: CM - carboxymethyl; AS - ammonium sulfate; DEAE - diethylaminoethane; DTT - dithiothreitol; BSA - bovine serum albumin; SN - supernatant; PTT - precipitate; $KPO_4$ - mixture of $K_2HPO_4$ and $KH_2PO_4$.

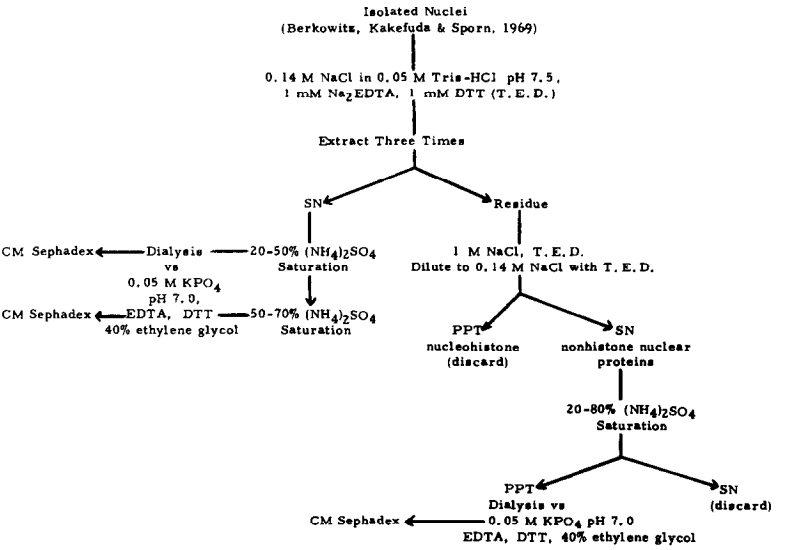


Figure 1: Summary of the extraction of DNases from purified HeLa nuclei.

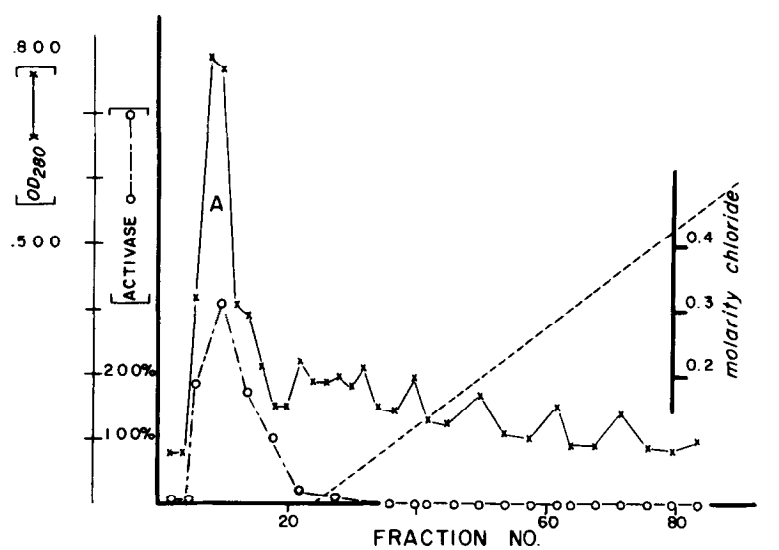


Figure 2: CM-Sephadex elution profile of the 20-80% AS fraction of the 1.0 M NaCl extract of HeLa nuclei. DNase activity was assayed by the "activase" method (14), and expressed as the % increase in priming activity over untreated native DNA.

retained by the column. The fraction of the nucleoplasmic extract which precipitated at 50-70% saturation with AS contained two DNase activities, $B_1$ and C, which were not completely separated by the salt gradient (Fig 3).

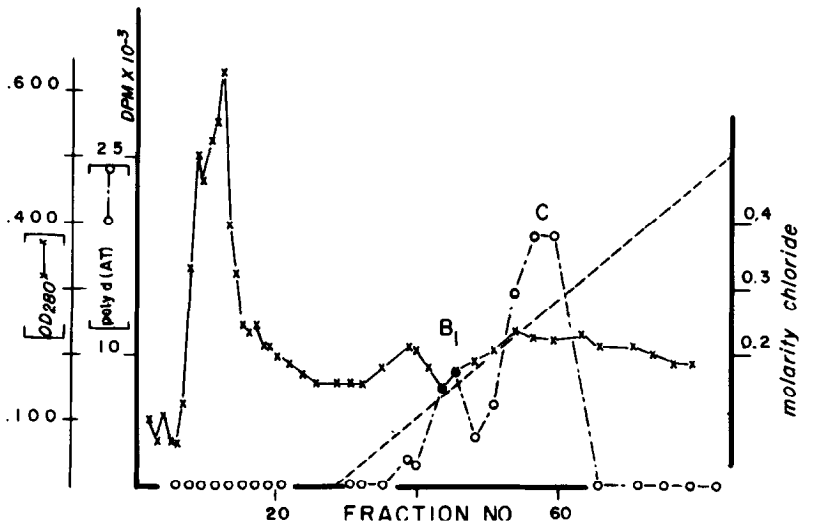


Figure 3: CM-Sephadex elution profile of the 50-70% AS fraction of the 0.14 M NaCl extract of HeLa nuclei. DNase assay reaction mixture contained: 6 x $10^4$ dpm poly d(A-T)-$^3$H; 50 mM glycine-NaOH pH 8.0; 2 mM $MgCl_2$; 2 mM DTT; 50 $\mu$g BSA; 50 $\mu$l column fraction. Total volume was 100 $\mu$l. The mixture was incubated at 37°C for 15 min and the reaction was terminated by the addition of 50 $\mu$l of 0.2% denatured calf thymus DNA, followed immediately by 50 $\mu$l of cold 14% $HClO_4$. DNase activity is expressed as the radioactivity in the supernatant shown on the ordinate labeled "poly d(A-T)".

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When these DNases were reapplied to CM-Sephadex separately only single peaks eluted; it was confirmed that elution occurs at 0.15 M NaCl for DNase $B_1$ and 0.25 M NaCl for DNase C.

The components of the nucleoplasmic extract which precipitate with 50% AS, also eluted from CM-Sephadex as two peaks of DNase activity (Fig 4). The first of these, marked $B_2$, was eluted by concentration of NaCl similar to that which displaced DNase $B_1$ from the column, and may be the same

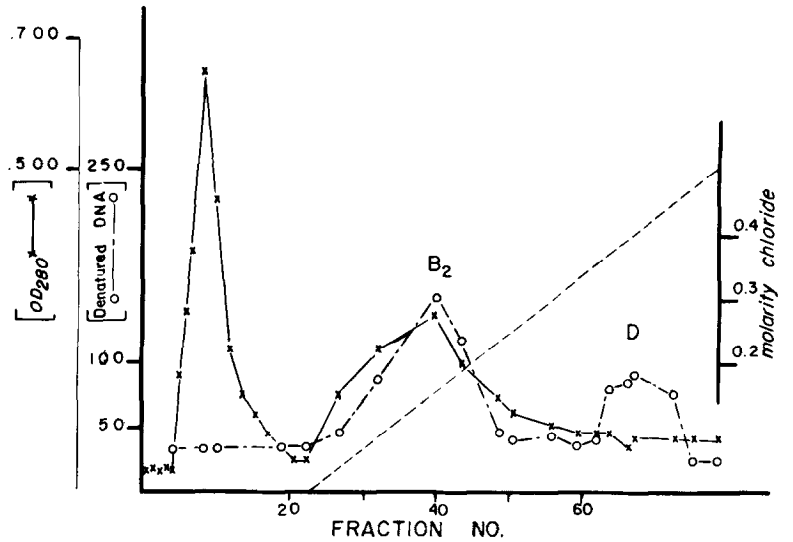


Figure 4: CM-Sephadex elution profile of the 20-50% AS fraction of the 0.14 M NaCl nuclear extract. DNase assay mixture contained, in a total volume of 0.4 ml: 300 $\mu$g heat denatured calf thymus DNA; 32 mM glycine-NaOH pH 8.0; 10 mM $MgSO_4$; 50 $\mu$l column fraction. Incubation at 47°C was for 30 min. The reaction was stopped by the addition of 0.2 ml of 0.6 N $HClO_4$, and A260 nm was read in the supernatant. Ordinate labeled "Denatured DNA" shows this A 260 nm x $10^3$.

enzyme. DNase D, however, showed high affinity for CM-Sephadex and 0.40 M NaCl was required to elute it.

The column effluent was assayed by methods which were most convenient for the particular case, but DNase activity could be demonstrated in each peak by any of the assays described in the Methods section. RNase and DNA polymerase activity could be detected only in peak B, but even here these activities disappeared when DNase B was purified further by DEAE-cellulose chromatography (Manuscript in preparation).

Temperature Stability of HeLa Nuclear DNases: Fig 5 shows that DNases C and D had thermal stabilities which were different from the stabilities of DNases A, $B_1$ and $B_2$.

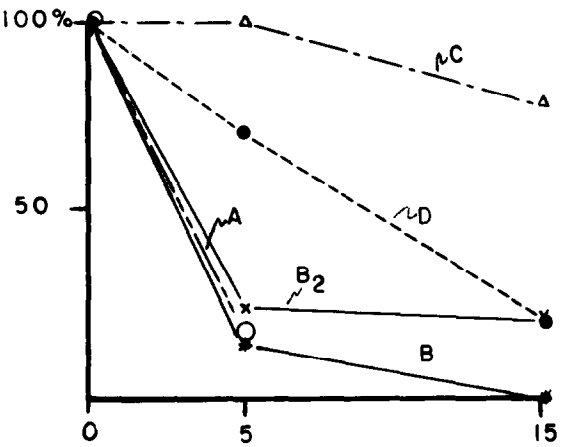


Figure 5: Temperature stability of HeLa nuclear DNases. The enzyme preparations eluted from CM-Sephadex were incubated at 45°C as indicated, and assayed by the "activase" method (14). Ordinate: percentage of unheated control; abscissa: time in min at 45°C prior to assay.

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Substrate Preferences: DNases A, $B_1$ and $B_2$ were again rather similar in that their activity was not greatly altered when the DNA substrate was denatured or irradiated with UV-light (Table 1). The activities of DNases C and D were markedly greater on denatured DNA, but DNase D was further characterized by its low ability to attack UV-irradiated DNA.

Ratio of "Activase" to Hydrolytic Activity: Table 2 shows that DNase A could be clearly distinguished from the other HeLa nuclear DNases by the fact that its ability to activate DNA for DNA polymerase, relative to its hydrolytic potential, was far greater than the ability of the other enzymes studied.

## DISCUSSION

This report provides the first demonstration that nuclei from a uniform and mycoplasma-free cell population contain at least four distinct DNases. Although speculations on the precise functions of these enzymes

TABLE 1

Relative Activities of HeLa Nuclear DNases on Native, Heat Denatured and UV-irradiated DNA.

| DNase | Native DNA | Denatured DNA | UV-irradiated DNA |
|---|---|---|---|
| A | 100 | 80 | 74 |
| $B_1$ | 100 | 72 | 75 |
| $B_2$ | 100 | 92 | 54 |
| C | 100 | 219 | 99 |
| D | 100 | 190 | 22 |

DNases were assayed by the ability to activate calf thymus DNA for subsequent DNA polymerase action (14).

TABLE 2

Ratio of DNA-activating to DNA-hydrolytic Activities of HeLa Nuclear DNases

| DNase | Activation of DNA/Hydrolysis of poly d(A-T)-$^3$H |
|---|---|
| A | 2.11 |
| $B_1$ | 0.21 |
| $B_2$ | 0.50 |
| C | 0.23 |
| D | 0.29 |

The procedures are described in the legend to Fig 3 and in reference 14.

must await their more exact characterization, it should be noted that our studies were performed on asynchronous cultures, so that some of these DNases could be characteristic of a particular stage of the cell cycle.

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